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NUMBER OF PAGES (INCLUDING COVER SHEET): 21

FROM: Jeff Lloyd 

DATE: October 22, 1993

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SUBJECT/MESSAGE: Re: Patent Application No. 07/713,624

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OCT 25 1993

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- U.S. Patent No. 4,448,885, issued May 15, 1984 to Schnepf *et al.* "*Bacillus thuringiensis* crystal protein in *E. coli*."
- U.S. Patent No. 4,467,036, issued August 21, 1984 to Schnepf *et al.* "*Bacillus thuringiensis* crystal protein in *Escherichia coli*."

THAT, I have reviewed Adang and Kemp application Serial No. 06/535,354 filed September 26, 1983, and the Office Action dated August 26, 1993; and being thus duly qualified, do further declare as follows:

*Bacillus thuringiensis* (B.t.) delta-endotoxins, by definition, are intracellular (i.e. cytoplasmic) proteins that accumulate as inclusion bodies. The protein subunits of the

In other words, *B.t.* delta-endotoxins are unlike the inclusion bodies of denatured forms of normally soluble proteins that are often found when overexpressed in bacteria. *B.t.* delta-endotoxins are found in inclusions, or crystals, when in their native conformation, awaiting appropriate conditions for activation. These properties are independent of protein size, relatedness, or target specificity. The variety of *B.t.* toxins known at the time included a size range from 28kDa to approximately 160kDa (Calabrese, D.M., Nickerson, K.W., and Lane, L.C. 1980. A comparison of protein crystal subunit sizes in *Bacillus thuringiensis*. Can. J. Microbiol. 26:1006-1010.; Tyrell, D.J., Bulla, L.A., Jr., Andrews, R.E. Jr., Kramer, K.J., Davidson, L.I., and Nordin, P. 1981. Comparative biochemistry of Selected *Bacillus thuringiensis* strains. J. Bacteriol. 145:1052-1062.), relatedness that existed at an ill-defined immunological level (see Tyrell *et al.*) and target specificity that clearly included diptera, lepidoptera, and unknown (e.g. Delucca, A.J., Simonsen, J., and Larson, A. 1979. Two new serovars of *Bacillus thuringiensis* : serovars, dakota, and indiana (serovars 15 and 16). J. Invertebrate Pathol. 34:323-324.). A brief discussion of immunological relatedness vs. activities among lepidopteran-active *B.t.s* is included in the Kronstad *et al.* reference.

- 1) They could be proteolytically degraded in the desired expression host;
- 2) They could require some *B.t.*-specific post-translational modification for useful activity (e.g. phosphorylation, glycosylation, etc.);

- 3) They could require some additional *B.t.*-specific proteins for attainment of an active conformation or assembly into an inclusion;
- 4) They could lack signals for transcription and translation in the desired expression host;
- 5) They could contain an "ill-conditioned" coding sequence or fortuitous DNA or RNA signals that are detrimental to expression due solely to their heterologous nature.

Of the reasons listed, topics 1 through 3 clearly relate to properties of the protein to be expressed, topic 4 relates to properties of the expression host and whether the "native" host of the protein-encoding gene has compatible gene expression signals, and topic 5 relates to general properties of genes of the "native" host not limited to the protein of interest.

At the time of the original application, all reported cloned crystal protein genes had been successfully expressed in *E. coli*, resulting in the production of insecticidally active proteins (e.g. Schnepf & Whiteley; Klier *et al.*). Owing to the differences in transcriptional regulation between *B.t.* delta-endotoxins and the average *E. coli* gene (addressed in Hofte & Whiteley, 1989; Schnepf and Whiteley, 1981; Klier *et al.*, 1982) detectable expression was, in fact, surprising. Therefore, topics 1-3 above were not a problem in the *E. coli* expression system. As explained below, genes naturally expressed in *E. coli* were known to be expressible in plants. Klier *et al.* (1982), even reported the production of inclusion bodies in *E. coli*. The latter observation indicates that the essential features of a delta-endotoxin are largely maintained using an essentially pure gene in a heterologous host.

Furthermore, the original application (Example 1, pp. 37-39) indicates that an incomplete gene is still capable of producing an insecticidal protein. Therefore production of an active protein is not particularly demanding, so long as the essential portion of the protein is encoded by the cloned gene.

Given knowledge at the time, one skilled in the art would have expected that if they could produce one delta-endotoxin protein in a plant and it would accumulate and be insecticidally active, that other delta-endotoxins would also accumulate and be active.

Subsequent work has confirmed that delta-endotoxin proteins can be expressed in a number of alternative hosts, and that essentially pure genes lead to the production of insecticidal proteins when properly configured for expression. The only exception to this is

the dependence of the *cytA* gene on a *B.t.* accessory protein for post-translational accumulation in *E. coli* but not *Bacillus subtilis* (reviewed in Hofte and Whiteley).

Regarding topic 4 above, the applicants were aware that gene expression in plants would require expression signals that differed from those required for expression in bacteria. In pages 17-19 of the application, the applicants are aware of the lack of expression of a number of genes of non-plant origin after transfer to plant cells, and they are careful to identify promoters, polyadenylation signals, etc. that are known to function in plants for expression of bacterial genes in plants. The products of these genes would be targeted to the cytoplasm of the plant cells rather than other cellular compartments.

Regarding topic 5 above, prior to expression of the *B.t.* toxin gene in plants, only bacterial genes of Gram negative origin had been expressed in plants, and these tended to have selectable markers: *nptII* (neomycin phosphotransferase) from transposon Tn5, conferring G418 resistance in plants; *dhfr* (methotrexate resistance) from Tn7; or were essentially marker enzymes: CAT (chloramphenicol resistance in bacteria) and octopine synthase from *Agrobacterium*. All of these genes were successfully expressed, however they were from bacteria with G+C contents of 50% or greater. *Bacillus thuringiensis* DNA however, was known to be about 34% to 40% G+C (e.g. Bergey's Manual of Determinative Bacteriology).

The DNA sequence requirements within the structural gene for efficient expression in plants were unknown at the time, however, it was known to be important for gene expression in yeast (J.L. Bennetzen and B.D. Hall, 1982. J. Biol. Chem. 257:3026) and might have been assumed to be applicable to other eukaryotic organisms. The difference in G+C content between *B.t.* genes and those that had already been expressed meant that they would at least differ from genes that had functioned successfully, possibly resulting in reduced translation. Additionally, it was known that the signal for polyA addition to eukaryotic mRNAs was "AAUAAA" (M. Fitzgerald and T. Schenk, 1981. Cell 24:251). Therefore, the likelihood of finding such a sequence inappropriately in a structural gene probabilistically increases both with the length of the gene and reduction of the G+C content (for DNA of 38% G+C content, this sequence could be expected to occur every 1100-1200 bases unless eliminated by evolution). The presence of such sequences could lead to truncated and inactive mRNA. Adventitiously encoded RNA splice signals might also be a barrier to

expression in plants, however, too little was known of the sequence requirements for splicing to predict how much of a hazard this might present to a *B.t.* gene.

All of the nucleic acid sequence-related problems that could cause difficulty in expression of *B.t.* toxin in plants would not be expected to be restricted to a particular *B.t.* toxin. Although the Hofte & Whiteley review proposed classification of *B.t.* endotoxin genes based on the type of insecticidal activity, and varying levels of amino acid sequence similarity were noted, the classification scheme does not depend on unique nucleic acid sequences. Rather, the nucleic acid sequence-related problems which could cause difficulty in expression of *B.t.* toxin in plants would be expected to be, and are, intrinsic properties of *B.t.* endotoxins across classes. In fact, they would be intrinsic properties of all *B.t.* genes encoding cytoplasmically (non-secreted) expressed proteins. If anything, as noted above, difficulty with expression due to these factors should correlate with sequence length. Therefore, expression of the initial toxin gene is a representative example for what would be expected for any *B.t.* endotoxin gene, or any other *B.t.* gene encoding a cytoplasmically accumulating protein.

The undersigned declares further that all statements made herein of his own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or of any patent issuing thereon.

Further declarant sayeth naught.

  
H. Ernest Schnepf

21 October 1993  
Date